Localization and Functional Mapping of AMPA Receptor Subunits in the Developing Rabbit Retina

Yun-Chieh Chang[1,2] *and Chuan-Chin Chiao*[1,3]

Abstract

PURPOSE. Glutamate has been suggested to regulate the development of retinal neurons, but ontogenic expression of ionotropic glutamate receptors has only recently been characterized in the rat retina. The purpose of this study was to examine the expression patterns of AMPA receptors and to functionally map glutamatergic drive in the developing rabbit retina.

METHODS. The retinas from New Zealand White rabbits of different developmental stages (embryonic days [E]21 and 26, postnatal days [P]0–10, and adult) were isolated and cryosectioned into vertical slices. Antibodies against GluR1, -R2/3, and -R4 were used to examine the postnatal expression patterns of the AMPA receptor subunits. To further map the glutamatergic drive in the developing rabbit retina, an agmatine (AGB)-activation assay was also used.

RESULTS. All AMPA receptor subunits, including GluR1, -R2/3, and -R4, were expressed in the inner plexiform layer as early as E26 and were convincingly labeled in the outer plexiform layer at P2. These AMPA subunits showed different spatial distribution and temporal expression patterns across the postnatal stages examined. The immunoreactivity of the AMPA subunits was weak at P0 to P2 and then showed a striking increase at P4 to P6. The AGB activation assay revealed that some amacrine and ganglion cells were activated with 2 μM AMPA as early as E26 and, in the presence of an increased concentration of AMPA (20 μM), some potential horizontal cells were activated at the same stage.

CONCLUSIONS. AMPA glutamate receptors express and function during the early stages of the developing rabbit retina, indicating that AMPA receptors are functional before synapse formation. The period of increasing expression pattern of AMPA subunits also coincides with the switch of the glutamatergic drive of the retinal wave and thus may contribute to the synaptic maturation in the retinal circuits.

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Glutamate is the most major excitatory neurotransmitter in the vertebrate retina. Photoreceptors, bipolar cells, and ganglion cells are known to release glutamate when mediating the vertical signal pathway in the adult retina.[1] Although previous studies have shown that glutamate appears in the neural blast layer during the embryonic stage of the rabbit retina well before the onset of synaptogenesis,[2, 3] the role of glutamate release in the establishment of retinal circuits during development is largely unknown.[4] Glutamate receptors are responsible for converting glutamate signals from presynaptic cells into responses at the postsynaptic cells.[5, 6] Therefore, an examination of the spatial and temporal appearance of glutamate receptors during the various developmental stages is crucial to an understanding of the involvement of the different types of glutamate receptors in retinal maturation.

Glutamate receptors are generally divided into two groups: ionotropic (iGluRs) and metabotropic (mGluRs).[7-9] iGluRs are ligand-gated cation channels that are composed of different subunits. Based on their pharmacologic and electrophysiological characteristics, iGluRs are subdivided into α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), kainate and *N*-methyl-D-aspartate (NMDA) receptors.[7, 10, 11] The AMPA receptors are tetrameric receptors made up of four subunits: GluR1 to -R4.[12] Previous studies have shown that differences in the subunit composition of these receptors determine the distinct functional properties of the AMPA receptors.[13,14] Thus, glutamate signaling during development may alter significantly, depending on the subunit composition of the AMPA receptors. To determine the importance of AMPA receptor heterogeneity in the mediation of retinal circuit maturation, it is important to characterize GluR subunit expression at the various different stages of development.

Recently, immunohistochemical studies of AMPA receptor distribution in the perinatal developing retina of rats have revealed an early expression of AMPA subunits in the IPL.[15-17] Both in situ hybridization and immunoblotting experiments have also shown that the expression of GluRs in the rodent retina is developmentally regulated.[18-20] These results indicate that AMPA receptors exist well before synapse formation in the inner retina. However, the functional roles of AMPA receptors in the developing retina remain unclear.

Although subunit localization studies provide spatial and temporal patterns of GluRs in the developing retina, the functionality of AMPA receptors cannot be inferred directly, because whether an AMPA receptor is functional may also depend on heteromeric subunit assembly.[9, 10] Agmatine (1-amino-4-guanidobutane; AGB), a cationic guanidinium analogue that permeates open cationic channels, was originally shown to be a useful marker of measuring cation fluxes in frog sympathetic ganglion cells.[21] In recent years, it has been established as a reliable tool for the examination of functional ionotropic glutamate receptors in the mammalian retina.[22-27] The AGB permeation technique provides high spatial resolution of the activated iGluRs in the adult retina[28] and has been applied to the study of iGluR functionality in the developing mouse retina.[29,30]

In the present study, we characterized the differential distribution and expression of the AMPA receptor subunits GluR1, -R2/3, and -R4 in the developing rabbit retina. The immunoreactivity of the GluR subunits was examined by confocal microscopy using subunit specific antibodies. Furthermore, we functionally mapped the AMPA receptors using the AGB assay at various development stages in the rabbit retina. The rabbit is one of the key species for studies of mammalian retinal organization, and the morphology and physiology of its retinal neurons are particularly well known.[31,32] Examining expression and function of

AMPA receptor subunits in the developing rabbit is crucial to understanding the significance of the ontogenesis of glutamate receptor in the mammalian retina. The results indicate that the AMPA glutamate receptors were expressed and were functional during the early stages of the developing rabbit retina. The period of increasing expression pattern of AMPA subunits also coincides with the switch of the glutamatergic drive of the retinal wave and thus may contribute to the synaptic maturation in the retinal circuits.[33] All results are discussed in the context of the role of AMPA receptors in the establishment of retinal circuitry.

Materials and Methods

Tissue Preparation

Retinas from New Zealand White rabbits at different developmental stages between embryonic day (E)21 and the adult stages were used. The day of birth was designated as postnatal day (P) 0. All rabbits were deeply anesthetized using a 1:1 mixture of ketamine (150 mg/kg) and xylazine (30 mg/kg). After enucleation and hemisection, the vitreous humor was removed, and the retina was carefully detached from the retinal pigment epithelium. The animal was then euthanatized with an overdose of ketamine. All procedures were approved by the institutional animal care and use committee and were conducted in accordance with the ARVO Statement for Use of Animals in Ophthalmic and Vision Research. The isolated retina was cut into pieces and then fixed in 4% paraformaldehyde and 0.01% glutaraldehyde in phosphate buffer (PB; 0.1M, pH 7.4) for 20 to 30 minutes at room temperature. After rinsing, which was followed by cryoprotection in 30% (wt/vol) sucrose in 0.1 M PB, the retinas were sectioned vertically in 12-μm slices with a cryostat. For the AGB assay, the retina pieces were incubated with AGB (described later) before fixation and cryosectioning. It is known that retinal neurons develop at different rates at different eccentricities.[34] To ensure similar sampling locations, an area between the central and midperipheral regions on the ventral side of the retina was chosen (excluding the far temporal and nasal sides, and typically at an eccentricity of 2 to 4 mm below the visual streak).

Immunohistochemistry

To reduce background staining, any nonspecific binding sites within the retinal slices were blocked by incubation with 4% normal donkey serum (Jackson ImmunoResearch Laboratory, West Grove, PA) in 0.1 M PB and 0.1% Triton X-100 for 1 hour at room temperature. After blocking, the samples were incubated separately with the primary antibodies against GluR1, -R2/3, and -R4 (Chemicon, Temecula, CA) for 48 hours at 4°C. The antibodies were diluted 1:100 in blocking solution. The polyclonal antibodies against GluR1, -R2/3, and -R4 were raised against synthetic peptides corresponding to the C-terminal sequences of rat glutamate receptor subunits in rabbits. All antibodies gave single bands of 106-, 110-, and 110-kDa molecular weight for GluR1, -2/3, and -R4, respectively, on Western blot analysis against the microsome fraction of the rat brain (manufacturer's technical information). In addition, we tested the specificity of these antiserums directly in our laboratory, and showed single bands of ~110 kDa molecular weight for GluR1, -R2/3, and -R4 on Western blot analysis against the rabbit brain tissue (Supplementary Fig. S1; all Supplementary Figures are online at http://www.iovs.org/cgi/content/full/49/12/5619/DC1). Furthermore, these antibodies also have been reported previously to label GluR1, -R2/3, and -R4 specifically in the rabbit retina.[35] To confirm the retinal layers and to label the AII

amacrine cells and some ganglion cells, we co-incubated the retinal slices with goat polyclonal antibody against calretinin (1:400; Chemicon). In a separate experiment, we also co-incubated the retinal slices with goat polyclonal antibody against choline acetyltransferase (ChAT, 1:200; Chemicon), to label cholinergic amacrine cells. After the specimens were rinsed, secondary antibodies conjugated to Cy5 and FITC (1:100; Jackson ImmunoResearch Laboratory) were applied overnight at 4°C to visualize the GluRs and calretinin, respectively. The specificity of the immunostaining was evaluated by omitting the primary antibody during the incubation steps (Supplementary Fig. S2). The retinal slices were finally mounted in the mounting medium containing 90% glycerol and 5% propyl gallate for confocal imaging. To ensure a direct comparison of the intensity of staining for the different AMPA receptor subunits, we performed immunohistochemistry at the same time on all samples, and all subsequent images were acquired with the same confocal settings. The problem of signal saturation in the immunocytochemistry applications was avoided by using lower dilution factors of primary antibody and carefully adjusting the confocal setting when taking images. The number of retinas per animal used at each developmental stage was E21 (2/2), E26 (4/4), P0 (6/6), P2 (6/6), P4 (4/4), P6 (5/5), P8 (5/5), P10 (5/5), and P25 (4/4).

AGB Activation

For the AGB functional mapping experiments, the retinas were first incubated in a modified physiological buffer.[36] The buffer was bubbled with 95% O_2/5% CO_2 for an hour before 25 mM AGB and two concentrations of glutamate agonist (2 and 20 μM AMPA) were added for the activation studies. These low and high concentrations of AMPA have been shown to activate different retinal neurons above the basal level in mice.[26] All incubations were performed for 6 minutes at 37°C. Retinal pieces were then fixed and cryosectioned as described earlier. Rabbit polyclonal AGB antibody (AB-1568; dilution 1:400; Chemicon) was used to visualize the activated cells. The rabbit antiserum was selective for AGB glutaraldehyde linked to bovine serum albumin, as determined by dot immunoassays (AB-1568; Chemicon). The detailed immunohistochemical procedure used for the AGB activation assay has been described previously.[22,24-26,29] There was no cross-reactivity shown against arginine, glutamate, and other amino acids (manufacturer's technical information). When the adult rabbit retinas were probed with anti-AGB antibody, there was no endogenous AGB signal, and AGB immunoreactivity was detected only in the retina when the incubation medium contained AGB.[25] To ensure selective activation of the AMPA receptors, 100 μM of CNQX (AMPA antagonist) or 50 μM of AP5 (NMDA antagonist) was co-incubated with AGB and 20 μM AMPA in a control experiment. The retinal slices were then mounted in the mounting medium containing 90% glycerol and 5% propyl gallate for confocal imaging. All reagents used in the AGB activation assay, including AGB, AMPA, CNQX, and AP5 were obtained from Sigma-Aldrich Corp. (St. Louis, MO). The number retinas per animal retina used at each developmental stage was E21 (2/2), E26 (2/2), P0 (3/3), P2 (4/4), P4 (3/3), P6 (3/3), P8 (4/4), P10 (3/3), and P25 (3/3).

Confocal Microscopy and Image Acquisition

All images were acquired with a confocal scanning module (LSM 5 Pascal; Carl Zeiss Meditec, Dublin, CA) mounted on a fluorescence microscope (Axioskop 2 Plus Mot; Carl Zeiss Meditec). A 40× objective lens (Plan-Neofluar, 0.75 NA; Carl Zeiss Meditec) was used, and a single optic slice less than 1.5 μm was obtained. Phase-contrast images were acquired

after confocal scanning to identify the retinal layers, and image intensity and contrast were adjusted (Photoshop; Adobe Systems, Mountain View, CA).

Results

The AMPA receptor subunits GluR1, -R2/3, and -R4 were present in the rabbit retina from E26 but showed different spatial distributions and temporal expression patterns throughout the postnatal stages analyzed. Most AMPA receptor subunits were found in both the inner and outer plexiform layers (IPL and OPL). Based on the results of the AGB activation assay, the AMPA receptors identified in the immunohistochemical study were confirmed to be functional from E26 in the rabbit retina.

Expression of AMPA Receptor Subunits in the Adult Rabbit Retina

All AMPA receptor subunits (GluR1, -R2/3, and -R4) have been shown to express in both the IPL and OPL in the adult rabbit retina.[35, 37-40] To confirm their expression patterns in the retinal slices, antibodies against the AMPA subunits were used to characterize their localizations in the retinal layers. Figure 1 shows confocal images of double-labeling of the AMPA subunits GluR1, -R2/3, or -R4 (red) and calretinin (green). Calretinin is known to label AII amacrine cells and some ganglion cells in the rabbit retina.[41-43] The immunoreactivity for GluR1 was abundant in the IPL and weak in the OPL. In contrast, the expression of GluR2/3 was apparent in both the IPL and OPL and also within some neurons in the ganglion cell layer (GCL). Similarly, GluR4 was strongly expressed in both the IPL and OPL. This immunolabeling pattern of the GluRs in the retinal slices of adult rabbit is consistent with previous results with the same antibodies.[38]

Expression of AMPA Receptor Subunits in the Developing Rabbit Retina

Confocal images of GluR1 subunit expression in the rabbit retina at different developmental stages are shown in Figure 2 . The GluR1 immunoreactivity was absent at E21 (Fig. 2A) , but was detectable in the IPL and the GCL at E26 (Fig. 2) .

After birth, GluR1 expression was found to be prominent in the IPL at P0 (Fig. 2C)and expression of GluR1 in the OPL was first detectable at P2 (Fig. 2D) . The immunoreactivity of GluR1 was most abundant in both IPL and OPL at P4 and P6.(Figs. 2E, 2F) . However, GluR1 expression became slightly reduced at P8 (Fig. 2G)and had reached the lower adult level at P10 (Fig. 2H) . The strength of GluR1 immunoreactivity was also quantified at all developmental stages (Supplementary Fig. S3), and the trend was generally consistent with the one shown in Figure 2 . This GluR1 expression pattern in the developing rabbit retina is dramatically different from that in the developing rat retina, where GluR1 immunoreactivity was never observed in the OPL in Brown Norway rats,[15] although GluR1 expression could be detected at P14 in White Wistar rats.[16] In the INL, unlike the rat retina, there was no colocalization of GluR1 expression and AII amacrine cells (calretinin immunoreactive neurons) at any of the postnatal stages (data not shown), which is consistent with a previous finding that GluR1 is not present at the synapses between rod bipolar cells and AII amacrine cells.[35]

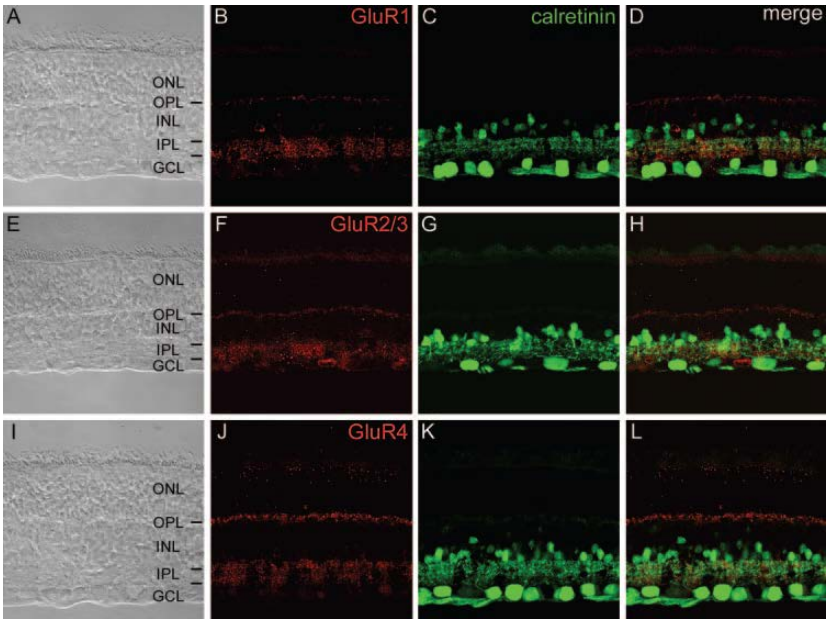

FIGURE 1. Expression patterns of AMPA subunits GluR1, -R2/3, and -R4 in the adult rabbit retina. (**A**–**D**) Confocal images show double labeling of the expression of the AMPA receptor subunit GluR1 (*red*) and calretinin (*green*). The phase-contrast image is shown in (**A**) to identify the retinal layers, and the merged image in (**D**) illustrates the colocalization of calretinin-positive cells (AII amacrine cells and some ganglion cells) and GluR1 expression. GluR1 immunoreactivity was abundant in the inner plexiform layer (IPL) and weak in the outer plexiform layer (OPL). (**E**–**H**) Similar to GluR1, GluR2/3 was expressed in both IPL and OPL and also in some neurons in the GCL. (**I**–**L**) Similar to GluR1, GluR4 was strongly expressed in both the IPL and OPL. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar, 20 μm.

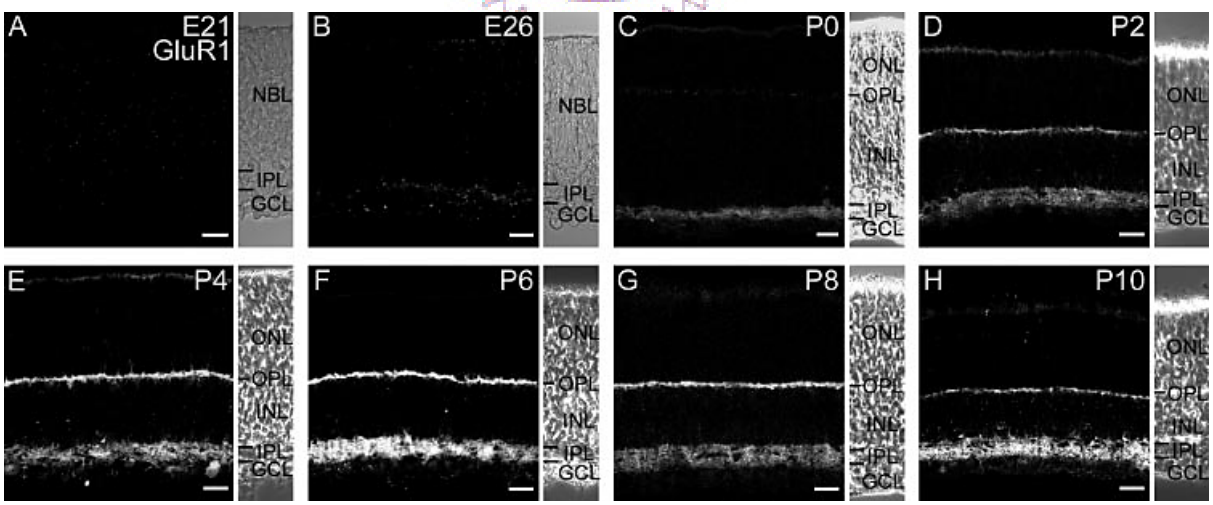

FIGURE 2. Localization of the AMPA subunit GluR1 in the rabbit retina at different developmental stages. (**A**) No GluR1 immunoreactivity was present at E21. (**B**) The expression of GluR1 was first identified in the inner retina at E26. (**C**) GluR1 was expressed strongly in the IPL after P0. (**D**) The expression of GluR1 in the OPL was first labeled at P2. (**E**–**F**) The immunoreactivity of GluR1 was most abundant in both the IPL and the OPL at P4 to P6. (**G**–**H**) The expression of GluR1 was slightly reduced at P8 to P10. NBL, neuroblastic layer. Scale bar, 20 μm.

Confocal images of GluR2/3 subunit expression in the rabbit retina at different

developmental stages are shown in Figure 3 . The immunoreactivity of GluR2/3 was barely detectable in some neurons of the inner retina at E21 (Fig. 3A) and labeling became moderate in the GCL at E26 (Fig. 3B) . GluR2/3 was steadily labeled in the IPL and weakly labeled in the OPL at P0 (Fig. 3C) , and the immunoreactivity was consistently labeled in both IPL and OPL at P2 (Fig. 3D) . Note that the GluR2/3 immunoreactivity was concentrated in two bands in the IPL at P2, in which the lower band coincides with the processes of the calretinin labeled cells (data not shown). From P4 to P8, GluR2/3 was strongly expressed in both IPL and OPL (Figs. 3E 3F 3G) . However, GluR2/3 expression was reduced and had reached the adult level at P10 (Fig. 3H) . The GluR2/3 immunoreactivity strength was also quantified at all developmental stages (Supplementary Fig. S3), and it was similar to the one shown in Figure 3 . The expression pattern of GluR2/3 in the developing rabbit retina is similar to that in the developing rat retina, though the immunostaining in both the IPL and OPL was detected earlier in rabbits.[15-17] The fact that the immunoreactivity of GluR2/3 shows two bands in the IPL throughout the developmental stages implies that GluR2/3 is expressed early in both cholinergic amacrine cells[38,44] and AII amacrine cells,[35,37] which is similar to the adult rabbit retina.

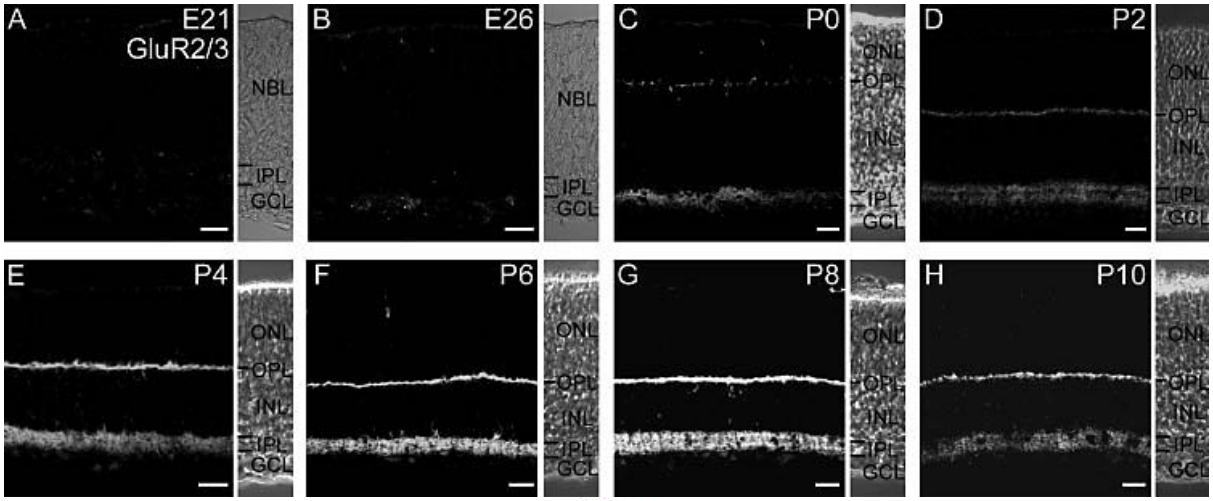

FIGURE 3. Localization of the AMPA subunit GluR2/3 in the rabbit retina at different developmental stages. (**A**) The expression of GluR2/3 was faintly labeled in some neurons of the inner retina at E21. (**B**) The immunoreactivity of GluR2/3 was moderate in the GCL at E26. (**C**) GluR2/3 was steadily labeled in the IPL and weakly labeled in the OPL at P0. (**D**) GluR2/3 was consistently labeled in both the IPL and the OPL at P2. (**E**–**G**) GluR2/3 immunoreactivity was most abundant in both the IPL and OPL at P4 to P8. (**H**) The expression of GluR2/3 was slightly reduced and reached an adult level at P10. Scale bar, 20 μm.

Confocal images of GluR4 subunit expression in the rabbit retina at different developmental stages are shown in Figure 4 . Similar to the GluR2/3 immunoreactivity found during the embryonic stages, GluR4 expression was barely detectable at E21 (Fig. 4A) and moderately labeled in both the IPL and GCL at E26 (Fig. 4B) . GluR4 immunoreactivity was also weakly detected in both the IPL and OPL at P0 (Fig. 4C) . In contrast to the GluR2/3 immunoreactivity, GluR4 expression in the OPL was strong at P2 to P4, whereas its immunostaining in the IPL remained moderate (Figs. 4D, 4E) . The immunoreactivity of GluR4 in the IPL did not reach its highest level until P6 to P8, indicating a late expression pattern compared with other AMPA receptor subunits (Figs. 4F, 4G) . The expression of GluR4 in both the IPL and OPL reached the adult level at P10 (Fig. 4H) . The strength of GluR4 immunoreactivity was also determined at all developmental stages (Supplementary

Fig. S3), and the trend matched to the one shown in Figure 4 . Overall, the expression pattern of GluR4 in the developing rabbit retina is similar to that of GluR2/3 and shows equivalent immunoreactivity in both the IPL and OPL in the developing rat retina.[15,16] Analogous to GluR2/3, there were two bands of GluR4 immunoreactivity discernible in the IPL beginning on P2, which corresponds to GluR4 expression in cholinergic amacrine cells[44] and AII amacrine cells[35] in the adult rabbit retina, which indicates that this specific pattern of expression is already present at early developmental stages.

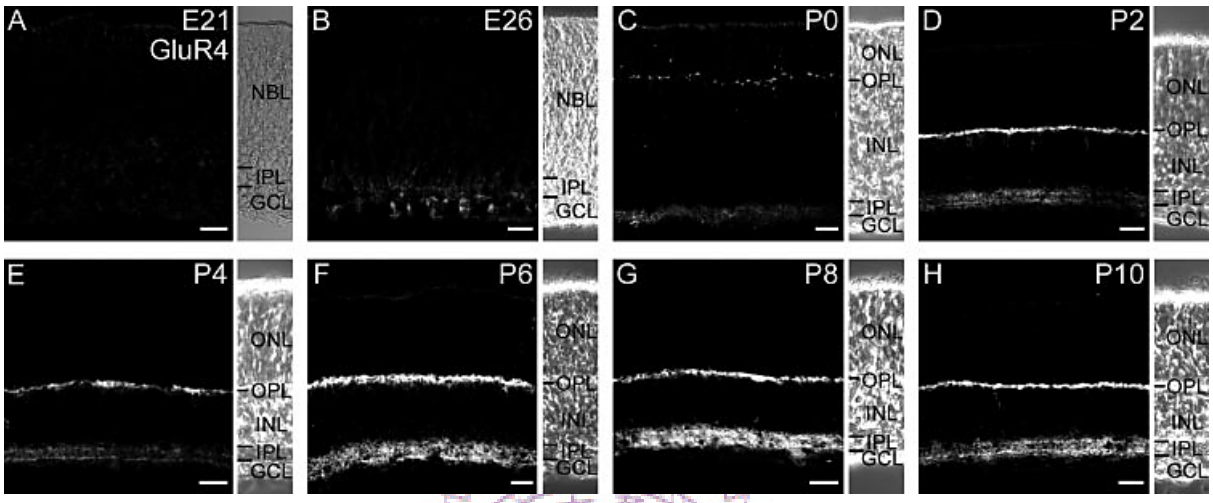

FIGURE 4. Localization of AMPA subunit GluR4 in the rabbit retina at different developmental stages. (**A**) Very weak immunoreactivity could be found in the inner retina at E21. (**B**) The expression of GluR4 was moderate in both the IPL and GCL at E26. (**C**) GluR4 was steadily labeled in the IPL and weakly labeled in the OPL at P0. (**D**–**E**) GluR4 immunoreactivity was first strongly detected in the OPL at P2, but remained moderate in the IPL at P2 to P4. (**F**–**H**) The expression of GluR4 in both the IPL and OPL was strong throughout P6 to P8 and reached an adult level at P10. Scale bar, 20 μm.

Functional Mapping of AMPA Receptors in the Developing Rabbit Retina

Functional AMPA receptors were probed by the AGB assay. Similar to the AGB permeation patterns in the adult rabbit retina,[25] we found that no endogenous AGB signal was observed when the retina was incubated in Edwards medium without AGB (Fig. 5A) , and there was a basal AGB permeation after incubation with 25 mM AGB in the absence of glutamate receptor agonists (Fig. 5B) . AGB permeation in the presence of 2 μM AMPA significantly increased in the neurons of the outer retina (horizontal cells, bipolar cells, and cone photoreceptors), as well as in some amacrine cells and ganglion cells of the inner retina (Fig. 5C) . With a high concentration of AMPA (20 μM), AGB permeation further increased in those cells in both inner and outer retinas (Fig. 5D) . This shows that the AGB signal activated by the AMPA in the adult rabbit retina is dose dependent. It was noted that the AGB immunoreactivity in the presence of AMPA showed two distinct bands in the IPL (Figs. 5C, 5D) , which colocalized with the ChAT bands (Supplementary Fig. S4) and indicates that the AMPA receptors are dominant on the cholinergic amacrine cells of the rabbit retina. To examine if the AGB signal activated by AMPA is also agonist specific, we cotreated 20 μM AMPA with 100 μM CNQX (AMPA/kainate receptor antagonist) and found that AGB signals were drastically reduced to the level of basal AGB permeation (Fig. 5E) . However, when we cotreated 20 μM AMPA with 50 μM AP5 (NMDA receptor antagonist), the AGB permeation was similar to that with 20 μM AMPA alone (Fig. 5F) .

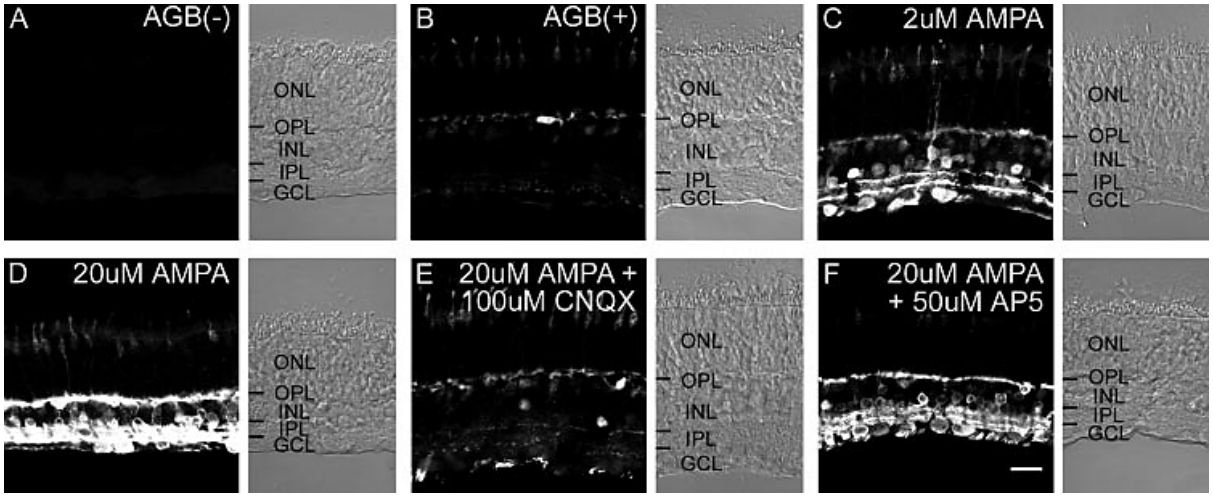

FIGURE 5. AGB signals in the adult rabbit retina activated by AMPA was dose dependent and agonist specific. (**A**) No endogenous AGB signal was observed when the retina was incubated in Edwards medium without AGB. (**B**) Basal AGB permeation after incubating the retina with 25 mM AGB in the absence of glutamate receptor agonists. There were endogenous AGB signals in some horizontal cells and bipolar cells, as well as cone photoreceptor cells in the outer retina and ganglion cells in the inner retina. (**C**) AGB signals in the presence of 2 μM AMPA. The low concentration of AMPA increased AGB permeation in horizontal cells, bipolar cells, and cone photoreceptors of the outer retina. More important, 2 μM AMPA significantly increased AGB signals in some amacrine cells and ganglion cells in the inner retina. (**D**) AGB signals in the presence of 20 μM AMPA. The high concentration of AMPA further increased AGB permeation in horizontal and bipolar cells, as well as amacrine cells and ganglion cells. (**E**) Cotreatment with 20 μM AMPA and 100 μM CNQX (the AMPA/kainate receptor antagonist) drastically reduced AGB signals to the basal AGB permeation. (**F**) Cotreatment with 20 μM AMPA and 50 μM AP5 (the NMDA receptor antagonist) had no effect on AGB permeation. Scale bar, 20 μm.

To characterize basal AGB permeation in the developing rabbit retina, Figure 6 shows AGB immunoreactivity at the various postnatal stages with or without AGB treatment. Throughout all the developmental stages analyzed, there was no endogenous AGB signal (Figs. 6A-D) . This result is identical with that found in the adult retina (Fig. 5A) . When the retina was incubated with 25 mM AGB, a basal level of AGB permeation was seen in the absence of glutamate receptor agonists in both the embryonic and the postnatal stages (Figs. 6E-H) . This basal AGB permeation pattern in the developing retina is similar to the one observed in the adult retina (Fig. 5B) . To ensure that the AGB signal activated by AMPA is also agonist specific in the developing retina, we cotreated 20 μM AMPA with 100 μM CNQX and found that AGB signals were drastically reduced to the basal level, but it did not completely abolish the basal AGB uptake (Supplementary Fig. S5).

The functional mapping experiment revealed that 2 μM AMPA could consistently activate AMPA receptors in amacrine cells and ganglion cells as early as E26, though a few AGB signals were detectable in some neurons at E21 (Figs. 7A, 7B) . This observation indicates that the expression of AMPA receptor subunits found at E26 was indeed functional and preceded synapse formation in the IPL. It should be noted that the AGB immunoreactivity was evident in a few immature neurons in the outermost part of neuroblastic layer (NBL) when activated with 2 μM AMPA at E26, but these AGB signals reduced significantly after birth. The AGB permeation pattern showed two conspicuous bands in the IPL at P0 (Fig. 7C) . The AGB signals also appeared in some amacrine and ganglion cells at the same age (see calretinin immunoreactive cells in Supplementary Fig. S6). The two bands of AGB signal persisted throughout all postnatal stage (Figs. 7C-H) . By colabeling ChAT with 2 μM AMPA activated AGB signals in all postnatal stages, we confirmed that

functional AMPA receptors were mainly localized in the cholinergic amacrine cells during development (Supplementary Fig. S4). The AGB immunoreactivity in the OPL appeared to express predominately in horizontal cells from the P0 stage onward (Figs. 7C-H) , though some bipolar cells were labeled at a later stage (e.g., P10). The overall AGB permeation pattern gradually increased its strength after birth and had reached the adult level at P10 (Figs. 7C- H) .

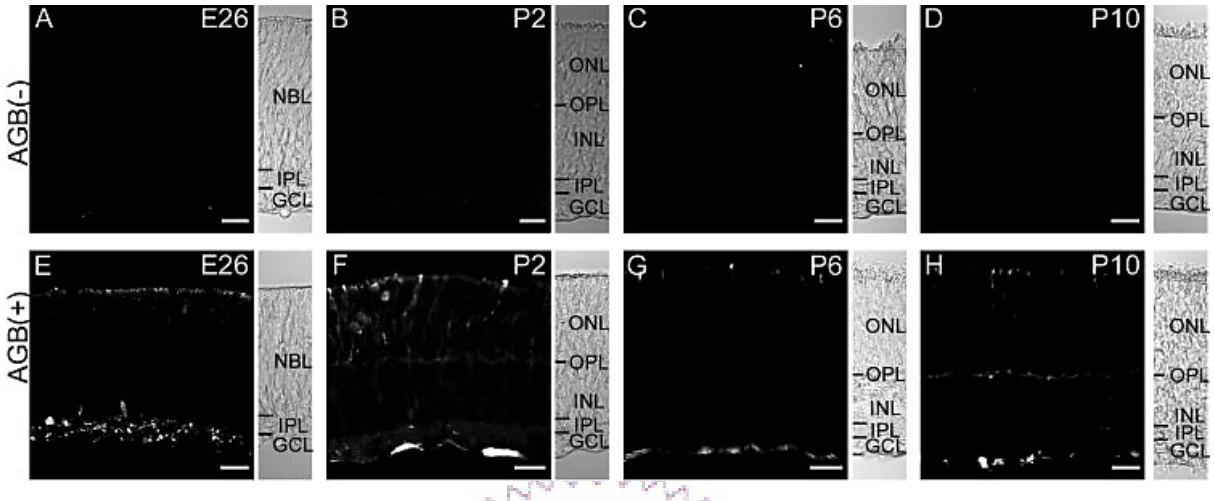

FIGURE 6. Basal AGB permeation in the rabbit retina at different developmental stages. (**A**–**D**) No endogenous AGB signal was observed when retinas from E26 to P10 were incubated in Edwards medium without AGB. (**E**–**H**) Basal AGB permeation after incubating the retinas from E26 to P10 with 25 mM AGB in the absence of glutamate receptor agonists. Endogenous AGB signals were detected in the inner retina and the outer margin of NBL at E26. At P2, there was a slight increase in AGB permeation in the ONL, but endogenous AGB was decreased at P6 and reached an adult level at P10. Scale bar, 20 μm.

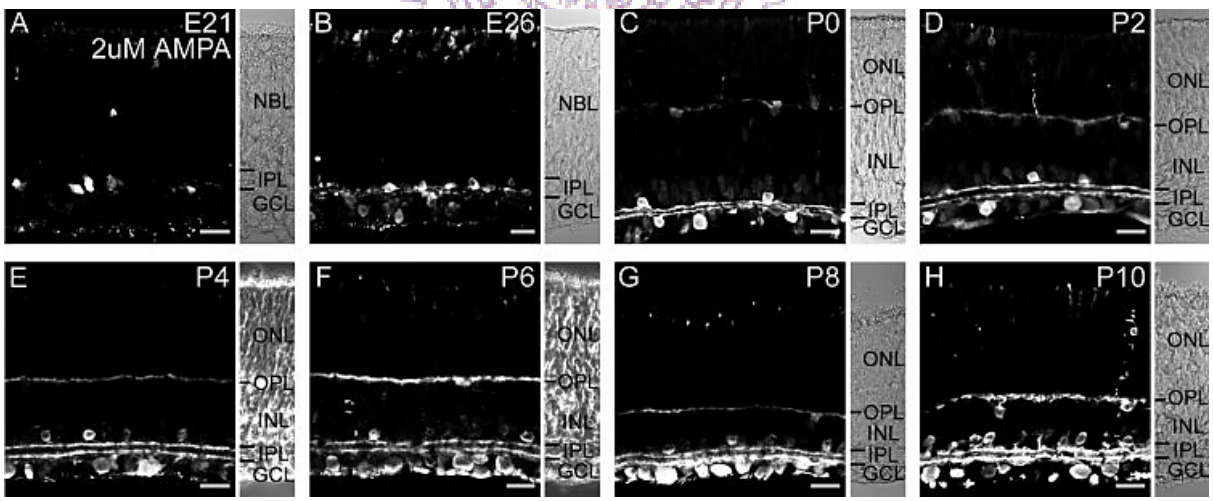

FIGURE 7. AGB signals activated by 2 μM AMPA in the rabbit retina at different developmental stages. The retinas were incubated with 25 mM AGB in the presence of 2 μM AMPA. (**A**) Some AGB signals were detectable in the inner retina at E21. (**B**) AGB permeation was found in some amacrine cells and ganglion cells, as well as the outer margin of NBL at E26. (**C**–**H**) After birth (P0–P10), the AGB signals were clearly detectable and steadily increased in some amacrine cells and ganglion cells in the inner retina and exhibited two distinct bands in the IPL. AGB permeation was weakly labeled in the OPL at P0 to P4, but showed a significant increase at P6 and reached an adult level at P10. Scale bar, 20 μm.

At the higher concentration, 20 μM AMPA could activate a few more cells in the NBL and in the inner retina at E21 (Fig. 8A) . However, more prominent AGB signals and the two-band pattern in the IPL were reliably detected at E26 (Fig. 8B). This dose-dependent AMPA activation indicates that glutamate receptors with different compositions of AMPA subunits may have different sensitivities toward AGB permeation. After birth, AGB immunoreactivity was localized in the horizontal cells of the OPL, in the two bands of the IPL, and in some amacrine cells and ganglion cells after treatment with 20 μM AMPA (Fig. 8C , also see calretinin immunoreactive cells in Supplementary Fig. S7). Similar to the low concentration of AMPA treatment, the AGB signals increased in both IPL and OPL from the P0 stage onward (Figs. 8C-H) . At P10, the AGB immunoreactivity pattern had reached the adult level (compare Figs. 8H and 5D).

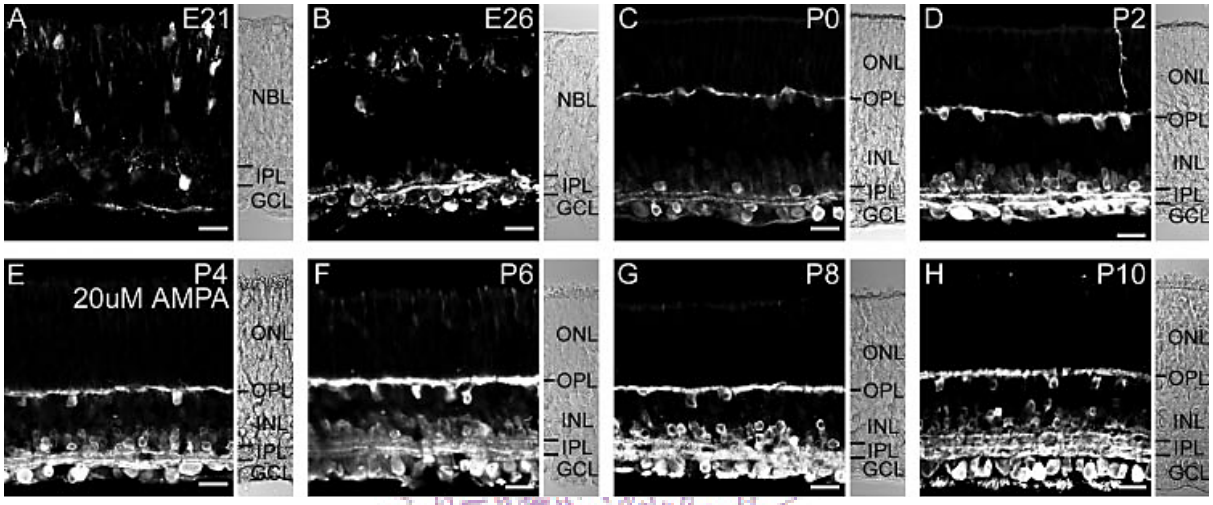

FIGURE 8. AGB signals activated by 20 μM AMPA in the rabbit retina at different developmental stages. The retinas were incubated with 25 mM AGB in the presence of 20 μM AMPA. (**A**) At high-AMPA concentration, AGB signals were detectable in both the inner retina and the NBL at E21. (**B**) AGB permeation was strongly labeled in some amacrine cells and ganglion cells, as well as the outer margin of NBL at E26, with activation of high AMPA concentration. (**C**–**H**) After birth (P0–P10), AGB signals with 20 μM AMPA were much stronger than with 2 μM AMPA in amacrine cells and ganglion cells. Similarly, AGB permeation also showed a significant increase in the OPL at P6 when activated with a high concentration of AMPA. AGB signals were adult like after P8. Scale bar, 20 μm.

Discussion

Glutamate and glutamate receptors are crucial for retinal development. In this study, we examined the expression in the developing rabbit retina of the AMPA glutamate receptor subunits GluR1, GluR2/3, and GluR4 by immunohistochemistry and mapped the functional AMPA receptors by the AGB activation assay. We found that all AMPA receptor subunits (GluR1, -2/3, and -R4) were expressed in the inner plexiform layer (IPL) as early as E26 and in the outer plexiform layer (OPL) at P2. Most of the immunoreactivity of the AMPA subunits was weak at P0 to P2 and showed a striking increase at P4 to P6. Nevertheless, different AMPA subunits showed differences in their spatial distribution and temporal expression patterns during the various developmental stages. The AGB activation assay revealed that some retinal neurons were activated with 2 μM AMPA as early as E26. Throughout the postnatal stages, functional AMPA receptors significantly increased and

reached adult level at P8 to P10. Taken together, these results indicate that AMPA glutamate receptors are functioning well before synaptogenesis and may contribute to circuit maturation in the developing retina. In the next section, we discuss functional roles of AMPA glutamate receptors at the various different stages of development.

Expression of AMPA Receptor Subunits in the Developing Retina

It has been shown that glutamate is present in the early stages of mammalian retinal development[2,3,45,46] and that glutamate signaling plays a crucial role in establishing specific circuits during retinal development.[47,48] Earlier studies using in situ hybridization revealed the expression pattern of AMPA receptor subunits in the mammalian retina.[49-51] Over the past decade, the localization of AMPA glutamate receptors in adult retinas have been intensively studied by immunohistochemistry.[52-61] In contrast to the wealth of data available for adult retinas, only a few studies have been performed to characterize the distribution of AMPA receptors in the developing retina and all of them have used rat retinas,[15-17] except one study of the developing chick retina.[62] We report the first evidence showing the expression of AMPA receptor subunits in the developing rabbit retina.

AMPA Receptor Subunits in the Outer Retina. Although the GluR1 subunit is predominately expressed in the dendrites of the OFF-cone bipolar cells in cat and rodent retinas,[55,63] previous studies have indicated that GluR1 expresses weakly in the OPL of the adult rabbit retina,[38]which is consistent with our immunostaining results (Fig. 1B) . Of interest, the immunoreactivity of GluR1 in the OPL was moderate at P2 (Fig. 2D) and reached its highest level at P6 (Fig. 2F) . This transient increase in GluR1 expression in the OPL during the first week of the postnatal stage implies that synapse formation between photoreceptors, the horizontal cells, and the OFF cone bipolar cells may require substantial functional AMPA receptors whose subunit composition involves GluR1. Although the expression of GluR2/3 and -R4 was stronger than that of GluR1 in the OPL of adult retinas (Figs. 1F, 1J) ,[38] the phenomenon of a transient increase in GluR2/3 and -R4 expression in the OPL from P2 to P8 (Figs. 3, 4) was similar to the expression pattern for GluR1 in the developing retina. It has been known that the beginning of ribbon synapse formation in the outer retina occurs at P2 to P6 in the developing rabbit retina.[64] Our finding of a transient increase in the AMPA subunits in the OPL during the first postnatal week supports the hypothesis that AMPA glutamate receptors may be involved in the synaptogenesis between photoreceptors, horizontal cells and bipolar cells during early retinal development. Taken together, this evidence indicates that the AMPA receptors and glutamate neurotransmission play an important role in the maturation of outer retina circuitry during development.[63,65]

AMPA Receptor Subunits in the Inner Retina. AMPA receptor subunits have been shown to express widely in different types of ganglion cells and amacrine cells in mouse retinas.[66] In adult rabbit retinas, previous studies have indicated that GluR2/3 and -R4 mainly express in AII amacrine cells,[35,37] cholinergic amacrine cells,[44] and directive selective ganglion cells.[38] The immunoreactivity of GluR2/3 and -R4 in the developing rabbit retina also showed corresponding bands in the IPL (Figs. 3, 4) . Similar to the transient increase of AMPA subunit expression in the developing OPL, all AMPA subunits showed increased immunoreactivity during the first week of the postnatal stage and reached an adult level after P10. Glutamate transmission has been indicated to be involved in dendritic remodeling of ganglion cells during development.[47,48,67,68] Our evidence that AMPA subunit expression

increases in the IPL during the early postnatal days supports the idea that glutamate transmission via AMPA receptors is required to refine the inner retina circuitry.[69]

Functionality of Glutamate Receptors in Retinal Development

Functional mapping of glutamate receptors by observing AGB entry secondary to agonist activation has been widely used to study the functionality of glutamate receptors in mammalian retinas.[22-26,28,70] The AGB permeation pattern activated by different glutamate agonists is comparable to the results obtained from electrophysiological experiments and neurotransmitter release studies.[24,25] Moreover, the immunocytochemically identified neurons and AGB gating patterns in the retina show a good correspondence to glutamate receptor distribution patterns.[26] Despite this great advantage, only one recent study has used this method to investigate glutamate receptor functionality in the developing mouse retina.[29] We report the first evidence of functional activation of AMPA glutamate receptors in the developing rabbit retina.

Localization of Functional AMPA Receptors. The appearance of functional AMPA receptors at E26 (Figs. 7B, 8B) is consistent with the earlier finding that diffuse glutamate labeling in the rabbit retina was detectable during various embryonic stages.[2,3,45] This also indicates that AMPA glutamate receptors are functional well before synaptogenesis in the developing retina and may contribute to the regulation of the neuronal cytoarchitecture and to cell migration.[71,72] In the mouse retina, it has been shown that AMPA receptors were expressed in embryonic retinal progenitor cells, and glutamate activation can regulate cell proliferation and cell fate specification.[73] Thus, the early expression of functional AMPA receptors found in the embryonic rabbit retina may be important for generating the correct proportion of retinal cell types during development. After birth, the AGB permeation pattern showed two conspicuous bands in the IPL when activated by both low and high concentrations of AMPA (Figs. 7, 8) and this result is similar to that of the adult retina (Fig. 5) . We have shown that these two bands correspond to the ChAT bands (dendritic processes of cholinergic amacrine cells) in the adult rabbit retina (Supplementary Fig. S4). We also confirmed that the two bands of AGB signals observed in the IPL also correspond to the ChAT bands during all postnatal stages of the developing rabbit retina (Supplementary Fig. S4). This implies that AMPA glutamate receptors are functionally dominated in the cholinergic amacrine cells throughout these developmental stages, although the expression pattern of all AMPA subunits (GluR1, -R2/3, and -R4) in the IPL of the developing rabbit retina does not form these two bands as distinctly (Figs. 2, 3, 4) .

AMPA Receptors and Cell Death. It has been shown that high levels of glutamate in the neonatal retina are critical for the regulation of the differential activation and remodeling of developing neurons.[3, 74] In retinal development, about half the population of ganglion cells dies by maturity.[34,71] In the developing rat retina, amacrine cells, and horizontal cells increase their death rate by 20% between P2 and P10.[75] It has been hypothesized that cell death through non-NMDA receptors plays a significant role in retinal maturation, possibly by selective Ca^{2+} permeation via the GluR2 subunits.[76-79] Our results on the functional expression of AMPA glutamate receptors in the neonatal rabbit retina support the idea that glutamate signaling though these early activated AMPA receptors is essential for the regulation of cell death and retinal circuit maturation, although programmed cell death modulated by NMDA receptor activation may be another dominant factor.[80,81]

Glutamate Activation and Retinal Wave. Previous studies have indicated that synchronized spontaneous activity in the developing retina (spontaneous retinal waves) is essential for many developmental events.[82,83] In the rabbit retina, the spontaneous waves show a dramatic and coordinated transition in the excitatory drive from a fast cholinergic to a fast glutamatergic input at P1 to P3.[33,84] After P3, the local excitation of the retinal waves is mainly mediated by glutamatergic transmission.[33] Our findings show that the AMPA subunit expression is transiently increased at P2 to P8 and that the high levels of AGB permeation secondary to AMPA activation after birth coincide well with this period of spontaneous retinal waves (stage III).[84] Thus, our results indicate that the AMPA glutamate receptors may play an important role in mediating this synchronized spontaneous activity during the postnatal stages. This activity is crucial for the synaptogenesis and retinal circuit maturation.

Ontogenesis of Glutamate Receptors and Retinal Degeneration. The expression of functional glutamate receptors is important for normal retinal development.[4] In recent years, it has been well recognized that the functional expression of glutamate receptors is significantly altered in rodent models of retinal degeneration.[85] In the *rd* mice, it is known that the expression levels of AMPA receptor subunit GluRs were increased during development.[86] It has also been reported that photoreceptor degeneration in the *rd1* mouse retina correlated with the glutamate-mediated excitotoxic mechanisms.[87] Furthermore, the increased expression of GluRs was also associated with neuronal degeneration observed in the retina of experimental glaucomatous rats.[88] Although the rabbit eye does not have the equivalent animal model of retinal degeneration, results of glutamate receptor ontogenesis described in the present study provide relevant evidence for examining the role of AMPA receptors in rodent models of retinal degeneration.

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